Plant adaptive radiation mediated by polyploid plasticity in transcriptomes

Shimizu-Inatsugi, Rie ; Terada, Aika ; Hirose, Kyosuke ; Kudoh, Hiroshi ; Sese, Jun ; Shimizu, Kentaro K

Abstract: The habitats of polyploid species are generally distinct from their parental species. Stebbins described polyploids as "general purpose genotypes," which can tolerate a wide range of environmental conditions. However, little is known about its molecular basis because of the complexity of polyploid genomes. We hypothesized that allopolyploid species might utilize the expression patterns of both parents depending on environments (polyploid plasticity hypothesis). We focused on hydrological niche segregation along fine-scale soil-moisture and waterlogging gradients. Two diploid species, Cardamine amara and C. hirsuta, grew best in submerged and unsubmerged conditions, respectively, consistent with their natural habitats. Interestingly, the allotetraploid C. flexuosa derived from them grew similarly in fluctuating as well as submerged and unsubmerged conditions, consistent with its wide environmental tolerance. A similar pattern was found in another species trio: allotetraploid C. scutata and its parents. Using the close relatedness of Cardamine and Arabidopsis, we quantified genome-wide expression patterns following dry and wet treatments using an Arabidopsis microarray. Hierarchical clustering analysis revealed that the expression pattern of C. flexuosa clustered with C. hirsuta in the dry condition and with C. amara in the wet condition, supporting our hypothesis. Furthermore, the induction levels of most genes in the allopolyploid were lower than in a specialist diploid species. This reflects a disadvantage of being allopolyploid arising from fixed heterozygosity. We propose that recurrent allopolyploid speciation along soil-moisture and waterlogging gradients confers niche differentiation and reproductive isolation simultaneously, and serves as a model for studying the molecular basis of ecological speciation and adaptive radiation.

DOI: https://doi.org/10.1111/mec.13738

Received Date : 25-Jan-2016

Revised Date : 27-May-2016

Accepted Date : 01-Jun-2016

Article type : Special Issue

Plant adaptive radiation mediated by polyploid plasticity in transcriptomes

Authors:

Rie Shimizu-Inatsugi (1), Aika Terada (2,3,4), Kyosuke Hirose (5), Hiroshi Kudoh (5), Jun Sese (4,6), Kentaro K. Shimizu (1,4,5,7)

1. Department of Evolutionary and Environmental Studies and Department of Plant Biology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland.

2. PRESTO, JST, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan.

3. Department of Computational Biology and Medical Science, Graduate School of Frontier Sciences, The University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8561, Japan.

4. Biotechnology Research Institute for Drug Discovery, AIST, 2-4-7 Aomi, Koto-ku, Tokyo 135-0064, Japan.

5. Center for Ecological Research, Kyoto University, Hirano 2-509-3, Otsu 520-2113, Japan.

6. Artificial Intelligence Research Center, AIST, 2-4-7 Aomi, Koto-ku, Tokyo 135-0064 Japan.

7. Kihara Institute for Biological Research, Yokohama City University, 641-12 Maioka, Totsuka-ward, Yokohama, Kanagawa 244-0813, Japan.

K*eywords*: advantages and disadvantages of polyploidization, ecological speciation, generalist, genome duplication, water-usage gradients, specialist

Corresponding author:

Kentaro K. Shimizu

Department of Evolutionary and Environmental Studies, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

Fax: +41 44 635 68 21

kentaro.shimizu@ieu.uzh.ch

Running title: **Ecological polyploid speciation of *Cardamine***

Abstract

The habitats of polyploid species are generally distinct from their parental species. Stebbins described polyploids as "general purpose genotypes," which can tolerate a wide range of environmental conditions. However, little is known about its molecular basis because of the complexity of polyploid genomes. We hypothesized that allopolyploid species might utilize the expression patterns of both parents depending on environments (polyploid plasticity hypothesis). We focused on hydrological niche segregation along fine-scale soil-moisture and waterlogging gradients. Two diploid species, *Cardamine amara* and *C. hirsuta*, grew best in submerged and unsubmerged conditions, respectively, consistent with their natural habitats. Interestingly, the allotetraploid *C. flexuosa* derived from them grew similarly in fluctuating as

well as submerged and unsubmerged conditions, consistent with its wide environmental tolerance. A similar pattern was found in another species trio: allotetraploid *C. scutata* and its parents. Using the close relatedness of *Cardamine* and *Arabidopsis*, we quantified genome-wide expression patterns following dry and wet treatments using an *Arabidopsis* microarray. Hierarchical clustering analysis revealed that the expression pattern of *C. flexuosa* clustered with *C. hirsuta* in the dry condition and with *C. amara* in the wet condition, supporting our hypothesis. Furthermore, the induction levels of most genes in the allopolyploid were lower than in a specialist diploid species. This reflects a disadvantage of being allopolyploid arising from fixed heterozygosity. We propose that recurrent allopolyploid speciation along soil-moisture and waterlogging gradients confers niche differentiation and reproductive isolation simultaneously, and serves as a model for studying the molecular basis of ecological speciation and adaptive radiation.

Introduction

Polyploid speciation is widespread in plants, fungi and animals (Levin 2002; Ramsey & Schemske 2002; Van de Peer *et al.* 2009). Approximately 15% of speciation events in flowering plants and 31% in ferns are estimated to be polyploid speciation (Wood *et al.* 2009). Polyploidization confers strong reproductive isolation from meiotic failure instantaneously, although a low frequency of gene flow might persist (Levin 2002; Ramsey & Schemske 2002). Polyploidization has been considered to affect phenotypes in two ways: by the effect of genome duplication itself such as larger cell size, and by that of combining parental genomes by hybridization. The latter is pronounced in allopolyploidization, or genome duplication with interspecific hybridization, but also is applicable to autopolyploidization, or that within a species, because auto- and allopolyploidization can be considered a continuum along the genetic distance between parental genotypes (Levin 2002; Soltis *et al.* 2010).

It is often suggested that new polyploids must undergo ecological speciation by colonizing a different ecological niche, otherwise new polyploids would disappear easily because of the disadvantage of having the minority cytotype (Levin 2002). In 1971, Stebbins summarized many cases where the distribution range of polyploids is distinct from and broader than that of diploids. He proposed that polyploids are "general purpose genotypes," which can tolerate a wide range of environmental conditions. Since then, differences between diploids and polyploids in tolerance to stresses, such as water exposure, temperature, nutrients and light have been studied (reviewed by Levin 2002; te Beest *et al.* 2012). A broader range of environmental tolerance is also found in human-induced polyploids, such as in domesticated wheat (Dubcovsky & Dvorak 2007). The effect often depends on species, as noted by Levin, who found "a moderate amount of anecdotal evidence that some polyploids are more tolerant of drought than are their diploid prototypes."

Hydrological niche segregation along fine-scale soil-moisture and waterlogging gradients is gaining increasing attention as a major mechanism of coexistence in species-rich plant communities (Silvertown *et al.* 1999; Silvertown *et al.* 2015). These gradients provide a large number of different ecological niches. Species with different soil moisture responses live close to each other along a steep gradient within a short distance from water bodies. A strong negative correlation between soil drying and waterlogging tolerance across species has been recognized as a major cause of the fine-scale niche separation in wetlands (Silvertown *et al.* 1999). Because these studies focused on phylogenetically diverse plant communities, little is known on how speciation occurs with potential gene flow within a short distance, nor on the molecular basis of species differences in soil-moisture and waterlogging responses.

Stress-induced gene expression changes play a major role in the soil drying and waterlogging tolerance of plants in conjunction with constitutive gene expression, developmental traits such as stomata density and physiological traits such as stomata closure. The transcriptomic changes in response to drought have been studied extensively using the model plant *Arabidopsis thaliana* (reviewed by Nakashima *et al.* 2014). Recent data on transcriptomic changes following submergence stress in *Arabidopsis* and *Rorippa* indicated that they are distinct from drought responses (Lee *et al.*

2011; Sasidharan *et al.* 2013). Although drought and submergence may be considered the two extremes of a single physical dimension of water availability, the responses of plants are distinct, and not simply up- and downregulation of the same gene sets (Voesenek & Bailey-Serres 2013).

The genus *Cardamine* has long been studied on polyploid evolution and the association of ploidy and ecological differences such as the degree of soil wetness (Howard 1948; Hussein 1948, Lihova & Marhold 2006). It is closely related to *A. thaliana*, and an *Arabidopsis* microarray has been applied successfully to the *Cardamine* species to study its transcriptomes (Morinaga *et al.* 2008; Tedder *et al.* 2015). It is one of the largest genera in the Brassicaceae with >200 species, majority of which are estimated to be polyploid (Carlsen *et al.* 2009; Kucera *et al.* 2005; Lihova & Marhold 2006). *Cardamine* also provides one of the rare documented cases of a contemporary polyploid speciation event that occurred during the past 150 years in Urnerboden, Switzerland (Urbanska *et al.* 1997), wherein the hybridization of diploid *C. amara* and diploid *C. rivularis* yielded the triploid *C. insueta*, and further hybridization with tetraploid *C. pratensis* yielded hexaploid and pentaploid *C. schulzii* (Mandakova *et al.* 2013; Zozomova-Lihova *et al.* 2014). These new polyploid plants are mainly found in man-made meadows, and differentiation in terms of the availability of both water and nutrients has been documented (Urbanska-Worytkiewicz & Landolt 1978). Here, we focus on two allopolyploid species and their deduced parental species (Fig. 1). The allotetraploid species *C. flexuosa* ($2n = 4x$) is derived from *C. amara* and *C. hirsuta* or their closely related taxa, as has been shown by chromosomal painting (Mandakova *et al.* 2014) and by the affinity of plastid DNA and ribosomal internal transcribed spacer (ITS) sequences to *C. amara* (Carlsen *et al.* 2009; Lihova *et al.* 2006a). *Cardamine amara* grows in wetland habitats where the subterranean parts are constantly submerged in Eurasia, and. *C. hirsuta* is found in fairly dry open fields (Grime *et al.* 2007; Yatsu *et al.* 2003) and is a model species for evolutionary developmental biology (evo–devo) studies (Hay *et al.* 2014). The derived tetraploid *C. flexuosa* has also been used in evo–devo studies (Zhou *et al.* 2013), and appears in shaded, rather wet environments, which rarely dry up (Grime *et al.* 2007; Yatsu *et al.* 2003). This species often coexists with *C. amara* or *C. hirsuta* in a short distance in Europe (Grime *et al.* 2007; Landolt 2001), and can be regarded as a relatively young allopolyploid species

(10^4 to 10^5 years old) (Mandakova *et al.* 2014). Similarly, *C. scutata* is a tetraploid ($2n = 4x$), and cpDNA and ITS sequences suggest that it is derived from *C. amara* and *C. parviflora* or their close relatives (Carlsen *et al.* 2009; Lihova *et al.* 2006a). While *C. parviflora* is found in moist open habitats in Eurasia and America (Lihova *et al.* 2006a), the allotetraploid *C. scutata* is found in ill-drained paddy fields, creeks or river margins in East Asia (Kimata 1993; Lihova *et al.* 2006a).

Here, we conducted growth experiments using the two allopolyploid species and their parental diploid species to determine whether the allopolyploid species grow in a broader range of hydrological environments, including fluctuating conditions. Next, we conducted transcriptomic studies with dry and wet treatments, and validated microarray results with quantitative polymerase chain reaction (qPCR) amplification. We hypothesized that the allopolyploids might utilize two transcriptomic patterns derived from each parent depending on the conditions, and tested this polyploid plasticity hypothesis in transcrtiptomes using clustering analysis. We further examined whether there is a disadvantage in being allopolyploid using transcriptome data. We propose that such allopolyploid speciation along soil-moisture and waterlogging gradients provides for niche differentiation and reproductive isolation simultaneously, and serves as a valid model for studying the molecular basis of ecological speciation and adaptive radiation by taking the advantage of the availability of parental taxa for experimental studies.

Methods

Growth experiments

All plants used in the growth experiments are listed in Supporting Information Table S1. Seeds were germinated on sand with water in plastic dishes, and small seedlings (cotyledon stage) were transferred to plastic pots (15.8 cm wide, 19.8 cm deep) filled with soil. The soil was a mixture of vermiculite (10 mm in diameter) and gardening soil (Kureha Corporation, N:P:K = 0.4:1.9:0.6 g per kg soil, respectively) at a ratio of 2:1. We incubated three seedlings in each pot—one from each of the

trio of species—to enable each of them to be subjected to equivalent growth conditions, and situated the pots in one of three conditions: an unsubmerged (rather dry) condition, where plants were watered with 20 ml/pot per day for 3 weeks and subsequently 20 ml/pot per week for 7 weeks; a submerged condition, where plants were submerged up to the soil surface in containers; and a fluctuating condition, where plants were switched between submerged and dry conditions every 2 weeks. We placed 12 pots in each treatment condition. The growth experiment was conducted in a glasshouse at Kobe University, Hyogo, Japan (34°43′44″ N, 135°14′04″ E, alt. c. 130 m) from December 3, 2007 to February 11, 2008 (10 weeks) to simulate the typical fall germination among the Brassicaceae. The average ambient temperature was 7.6 °C (range 2.0–13.6 °C measured using a HOBO U22 data logger; Onset, MA, USA) during this period.

At the end of the growth experiment, we measured leaf numbers per individual plant, longest leaf length (in cm), rosette width (in cm) and total biomass (dried terrestrial and subterranean parts). The data were analyzed using Tukey's honest significant difference (HSD) test using R software (v. 2.8.0; https://www.r-project.org).

Plants used for transcriptome analysis

All plants used for microarray analysis were grown in a climate chamber (150 μmol/m^2/s photosynthetically active radiation (PAR) at the pot surface level) with 16 h light (22 °C)/8 h dark (20 °C) and 60% relative humidity. In this experiment, mature plants before flowering were subjected to dry or wet treatment in the same chamber. As controls, terrestrial parts of plants were collected without treatment. Plants were cut at the hypocotyl and terrestrial parts were incubated in the chamber for 2 h as the dry treatment to be comparable with the expression data of many *Arabidopsis* experiments studying acute responses (e.g., Yamaguchi-Shinozaki & Shinozaki 1994). For the wet treatment, a whole plant in a soil pot was transferred into a water tub with air bubbling (25 cm water

depth) for 2 h, and the terrestrial part was collected. All plants used were siblings or clones. A *C. amara* individual collected from natural field was propagated clonally in the laboratory. *C. hirsuta* and *C. flexuosa* were self-pollinated in the laboratory for several generations and the sibling seeds were grown for microarray analysis.

Transcriptome analysis

RNA was first extracted from each plant using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). The RNA was further purified by RNeasy Plant Mini Kits (QIAGEN, Hilden, Germany) in combination with DNase I treatment (QIAGEN). The purified RNA was labeled with Quick Amp labeling kits (Agilent Technologies; Santa Clara, CA, USA) and hybridized to an *Arabidopsis* (V4) Gene Expression Microarray, 4x44 (Agilent Technologies). Each experiment was performed with three replicates.

Each array result was normalized to compare gene expression profiles between different experiments (Supporting Information Fig. S1). Expression levels were $\log_2$ transformed and subjected to quantile normalization in which the upper quantile value in each library was set to 10. Because the microarrays were not designed for our target species, we selected trustable probes that satisfied both of the following criteria: expression levels > 7 in at least one of the observations; and "presence" flags in all three species, allowing us to focus on conserved genes among all species. To associate the probes with gene annotations, The Arabidopsis Information Resource (TAIR) ftp server (ftp://ftp.arabidopsis.org/Microarrays/Agilent/; released July 29, 2009) was used. When one gene was measured with multiple probes, the average value was used as the expression level of the gene. We analyzed 10,620 genes (Supporting Information Table S2). To be conservative, we compared fold changes among species rather than absolute expression levels because the divergence of the three Cardamine species from Arabidopsis may be different in the oligonucleotides of the microarray and may affect the hybridization strength.

Analysis of up- and downregulated genes

Up- and downregulation of each gene in each species were calculated from the microarray data in dry and wet treatments independently. Because of the limited number of replicates, we followed the simple fold-change method to detect up- or down-regulated genes instead of ANOVA. For dry treatment, the gene was regarded as upregulated if the gene expression level was more than four times that in control. Similarly, the gene was regarded as downregulated in dry treatment if the expression level was less than one-fourth of that in control. For the wet treatment, the thresholds of fold change for up- and downregulated genes were set to 3 and 1/3, respectively, because gene expression changes in wet treatment were smaller than those in dry treatment conditions.

Gene ontogeny (GO) analysis with Gene set enrichment analysis (GSEA)

This was performed to detect GO categories associated with highly expressed genes in dry and wet treatments. The 10,620 genes were analyzed using Java GSEA 2.0.7 program (Subramanian *et al.* 2005) to compare the gene expression levels between the target and control conditions for every species. For the functions of genes, GO Slim annotation of TAIR (Berardini *et al.* 2004) (ftp://ftp.arabidopsis.org/Ontologies/Gene_Ontology/; downloaded on January 10, 2010) was used. The GO categories that were below the fourth level in the hierarchy and associated with 10–500 genes were selected. The GO categories with statistical significance (nominal p-value < 0.1) were listed.

Hierarchical clustering and bootstrap testing

Hierarchical clustering of expression profiles was performed for each of the dry and wet treatments. Log_2 fold change values from the control to the target conditions were used based on the mean results of biological replicates. The values were converted to Z-scores for each treatment and species. Those genes whose standard deviations over three species were ≥1.5 were selected to observe interspecies differences. In total, 129 and 61 genes were used for the clustering analysis for dry and wet conditions, respectively (Supporting Information Table S3), using Ward's hierarchical clustering method with Euclid distance.

Bootstrap testing with 10,000 iterations in each treatment was performed to evaluate the correctness of the dendrogram generated by hierarchical clustering. In each iteration, the same numbers of genes as in the original dataset (129 and 61 genes for dry and wet conditions, respectively) were sampled with repeated random sampling, and Z-scores of genes in each experiment were calculated. Euclid distances of Z-scores from *C. flexuosa* to the other two species were calculated based on the genes whose Z-scores of *C. amara* were 1.5 or more different from those of *C. hirsuta*.

Reverse transcription and quantitative polymerase chain reaction (qPCR)

The same RNA samples as used in the microarray experiment were used for qPCR. We selected 11 genes with significant expression changes shown by microarray analysis, under either dry or wet treatments, and one control gene (*ACT2*) to design common primer pairs for the three species at conserved regions (Supporting Information Table S4). Total RNA was reverse transcribed using High-Capacity RNA-to-cDNA kits (Thermo Fisher Scientific). Reactions were conducted using SYBR^{TM} Green PCR Master Mix (Thermo Fisher Scientific) at primer concentrations of 100 nM.

Results

Allopolyploid species (C. flexuosa *and* C. scutata) *grew similarly along soil-moisture and waterlogging gradients including a fluctuating environment*

To test whether *Cardamine* allopolyploids could grow in a wide range of environmental conditions along soil-moisture gradient, we conducted growth experiments using two trios of diploids and tetraploid (Fig. 1) in three different water conditions: submerged (i.e. the roots are constantly submerged), unsubmerged (rather dry) and fluctuating (altering between submerged and unsubmerged conditions), as described above. We first tested a trio of species: allotetraploid *C. flexuosa,* diploid *C.*

amara and diploid *C. hirsuta* (Fig. 2 A–C). We studied small seedlings that is known to be high vulnerable to stresses, because the initial growth is most critical for plant establishment (Silvertown *et al.* 2015). We measured four traits after 10 weeks of growth to compare the effect of all three conditions on each species. Figure 2 shows the leaf numbers; the diploid species *C. amara* grew best in the submerged condition, which is equivalent to its original habitat in terms of water availability, in comparison with the fluctuating and unsubmerged conditions. Another diploid parental species, *C. hirsuta*, grew in the opposite manner: best in the unsubmerged condition and worst in the submerged condition. This indicates that these species are specialized for submerged or unsubmerged conditions, respectively. In contrast, the allopolyploid species *C. flexuosa* performed similarly in all three conditions. Although this species performed slightly better in the unsubmerged than in the submerged condition, the growth in the fluctuating environment showed no significant difference from either that in submerged or unsubmerged condition.

The longest leaf length (Supporting Information Fig. S2) and rosette width (Supporting Information Fig. S3) showed similar patterns, in which the diploid *C. amara* and *C. hirsuta* performed as specialists, and *C. flexuosa* performed similarly in all three conditions. The biomass (Supporting Information Fig. S4) showed significant differences between treatments only for *C. hirsuta* (see discussion).

The same treatments were applied to another trio of species: allotetraploid *C. scutata*, diploid *C. amara* and diploid *C. parviflora*. The overall growth tended to be slower than that of the initial trio, and this probably resulted in weaker results (Fig. 2 D–F, Supporting Information Figs S5–7). Nevertheless, the directions of differences were very similar. In terms of leaf numbers, *C. amara* performed best in the submerged condition, *C. parviflora* best in the unsubmerged condition, and *C. scutata* generated similar leaf numbers in all three conditions (Fig. 2 D–F).

These results from two trios of species supports the hypothesis that allopolyploid plants can grow in a wide range of environments including fluctuating conditions, while the diploid species are specialized to submerged (wet) or unsubmerged (rather dry) conditions.

Transcriptomic changes following dry and wet treatments among the allopolyploid C. flexuosa*, diploid* C. amara *and diploid* C. hirsuta

We hypothesized that the molecular basis of the similar growth of the allopolyploids in a wide range of environments is exploitation of parental gene expression patterns depending on conditions. To test this polyploid plasticity hypothesis, we studied genome-wide gene expression patterns. By taking advantage of the close relationship between the genus *Cardamine* and *Arabidopsis*, we used *Arabidopsis* gene expression microarrays from Agilent Technologies. We used one of the trio of species used in the growth experiments, namely the allotetraploid *C. flexuosa* and the two diploids *C. amara* and *C. hirsuta*. We subjected the three species to 2 h of either dry or wet treatments, and compared the gene expression in the aerial part between untreated (control) and dry- or wet-treated plants. The treatment time was kept short so that we could observe clear stress responses in the early stages. Supporting Information Fig. S1 shows the pairwise plots of expression levels of each array. Despite the experiments were performed on the microarray of *Arabidopsis thaliana*, the figure indicates that gene expression levels were stably observed among biological replicates (Pearson's correlation coefficient >0.93). We used 10,620 genes (Supporting Information Table S2) for all analyses, as described in the Methods.

The upregulated and downregulated genes observed by dry (>4 and <1/4) and wet (>3 and <1/3) treatments partially overlapped between *C. amara* and *C. hirsuta* (Fig. 3). In the dry treatment, more than 400 genes were upregulated in each species, and 166 genes were upregulated in all three species. The genes that were induced in *C. amara* and *C. hirsuta* but not in *C. flexuosa* were a minor set (28 genes). By dry treatment, well-known drought-induced genes such as *RD17* (At1g20440), *RD26* (At4g27410), *LEA14* (At1g01470) and *LEA* family gene (At3g02480) were upregulated at high rates (Supporting Information Table S2) in all three species, suggesting that *C. amara* has not completely lost its capacity for response despite its preference for a wet habitat. In the wet treatment, most genes (398) were upregulated in *C. hirsuta*. In contrast, the number of genes upregulated more than 3-fold in *C. amara* was remarkably small (130), suggesting that short-term (2 h) wet treatment

did not affect *C. amara* too much, but was harsh for *C. hirsuta*. The *C. flexuosa* plants showed intermediate numbers of gene changes compared with their parents by wet treatment.

In contrast to drought tolerance, submergence tolerance in the Brassicaceae has not been well studied, except for some reports on hypoxia (Nakashima et al. 2014; Voesenek & Bailey-Serres 2013). We checked the induction of representative genes that are induced by hypoxia in *A. thaliana*. The most notable result was the induction of *AHB1* (or *GLB1*) (At2g16060), a hemoglobin class I gene, which enhances the survival rate during oxygen shortage in plants (Hunt *et al.* 2002). The expression level of *AHB1* was most highly induced in *C. amara* (more than 11-fold), and also induced in *C. hirsuta* and *C. flexuosa* (4.8-fold and 8.7-fold, respectively). Another notable tendency was that some genes associated with hypoxia were induced in *C. amara* and *C. flexuosa*, but not in *C. hirsuta*. For example, *ADH* (At1g77120), an alcohol dehydrogenase gene, was upregulated more than 3-fold in *C. amara* and *C. flexuosa*, but was not induced in *C. hirsuta*.

Experimental validation of the microarray results

This cross-species microarray result was validated using qPCR, with common primer pairs for all species (Fig. S8). We chose the *ACT2* gene as a control, and five (including 2 *LEA* genes mentioned avobe) and six genes to be tested for the dry and wet treatments, respectively. The results of the qPCR generally showed consistency to the cross-species microarray data. ($R^2 = 0.858$; Supporting Information Fig. S9), suggesting the high confidence of this cross-species microarray experiments.

Gene set enrichment analysis

To evaluate the changes in physiological status produced by the dry or wet treatments and to compare further the responses of the three species, GSEA analysis was conducted. GO categories including significant numbers of genes with upregulation by dry or wet treatment in each species are listed in Supporting Information Tables S5 and S6, repetively. For dry treatment, 151, 158 and 122 GO

categories were enriched in *C. amara*, *C. hirsuta* and *C. flexuosa*, respectively. For wet treatment, 63, 131, and 106 categories were enriched, respectively. The overlap of the numbers of categories among species is indicated in different color codes in Tables S5 and S6, as well as in Fig. S10. The overall pattern is similar to the venn diagrams in Fig. 3 with gene numbers (See the discussion section for details).

Hierarchical clustering analysis of the genes with significantly high expression changes

To test whether the allotetraploid *C. flexuosa* shows two parental transcriptome patterns depending on environmental conditions, we compared similarity of $\log_2$-fold changes among species for dry or wet treatments by hierarchical clustering (see the Methods section). The clustering analysis revealed that the gene expression pattern of *C. flexuosa* clustered with either of the two parents specialized for dry or wet habitats, depending on the treatment. Thus, the gene regulation pattern of *C. flexuosa* was closer to *C. hirsuta* after dry treatment, and closer to *C. amara* after wet treatment (Fig. 4). We evaluated these patterns by bootstrap test using randomly selected genes. The clustering pattern by dry treatment (*C. flexuosa* was closer to *C. hirsuta* than to *C. amara*) was supported with 99.5% probability. Similarly, the clustering pattern by wet treatment (*C. flexuosa* was closer to *C. amara* than to *C. hirsuta*) was supported with 81.2% probability. These high probabilities illustrate the flexible changes in gene expression patterns exhibited by the tetraploid *C. flexuosa*.

Expression levels of the allopolyploid C. flexuosa *were intermediate between the two parental species for most genes*

By checking the gene induction level produced by each treatment, we found a notable tendency. Many genes showed upregulation levels in *C. flexuosa* that were intermediate between those of *C. amara* and *C. hirsuta*. Therefore, we extracted the 500 most highly upregulated genes according to the averaged upregulation levels of three species in each of the dry and wet treatments. These genes were

arranged into six categories according to their order of expression level (Table 1). The first two categories, [*C. amara* > *C. flexuosa* > *C. hirsuta*] and [*C. hirsuta* > *C. flexuosa* > *C. amara*] (the pattern like *STZ* in Fig. S8), indicate that the upregulation level was intermediate in *C. flexuosa*. After dry treatment, there were 266 genes in these two categories (88 and 178, respectively). On the other hand, there were 78 genes showing the highest upregulation level in *C. flexuosa* for the third and fourth categories, [*C. flexuosa* > *C. amara* > *C. hirsuta*] and [*C. flexuosa* > *C. hirsuta* > *C. amara*] (the pattern like *LEA14* in Fig. S8), (34 and 44 genes respectively). There were 156 genes showing the lowest upregulation level in *C. flexuosa* for the fifth and sixth categories, [*C. amara* > *C. hirsuta*> *C. flexuosa*] and [*C. hirsuta* > *C. amara* > *C. flexuosa*] (76 and 80 genes, respectively). These numbers suggest that more than half of the top 500 genes showed intermediate upregulation levels in *C. flexuosa*. A slightly stronger tendency was observed after wet treatment, where 302 genes (94 and 208 in the first and second categories, respectively) showed an intermediate level in *C. flexuosa*, 96 genes (37 and 59 in the third and fourth categories, respectively) showed the highest level in *C. flexuosa*, and 102 genes (46 and 56 in the fifth and sixth categories, respectively) showed the lowest level in *C. flexuosa*.

Discussion

Allopolyploid species as generalists vs. diploid species as specialists along soil-moisture and waterlogging gradients

We conducted growth experiments in three hydrological conditions: submerged (in which subterranean parts were constantly wet), unsubmerged (rather dry with limited irrigation) and fluctuating conditions (alternating between the submerged and unsubmerged conditions; Fig. 2). The natural habitat of *C. amara* is constantly submerged, and accordingly it grew best in the submerged condition. The natural habitats of *C. hirsuta* and *C. parviflora* are unsubmerged, and they grew best in the unsubmerged condition. Interestingly, the allopolyploids *C. flexuosa* (derived from *C. amara* and

C. hirsuta) and *C. scutata* (derived from *C. amara* and *C. parviflora*) grew similarly in all three conditions, including the fluctuating one. The similar pattern in the two trios of species indicates that diploid species are specialized either for submerged or unsubmerged condition, whereas the allopolyploid species performed as generalists with similar growth in all three conditions, including the fluctuating one. This supports the hypothesis that allopolyploid plants can obtain new hydrological niches along soil-moisture and waterlogging gradients that are different from their parental species, and can exploit fluctuating conditions successfully.

Among the four measured traits of initial growth, leaf numbers showed the most significant differences among conditions, followed by length of the longest leaf and rosette width. Biomass showed a significant difference only for *C. hirsuta*, possibly because its variance was higher than the other parameters and the duration of the experiment was insufficient to detect significant differences in the other species.

The effect of polyploidization on drought tolerance has long been discussed (reviewed by Levin 2002; te Beest *et al.* 2012). It has often been suggested that polyploid species have higher drought tolerance based on a comparison of autopolyploid and diploid, but the trend depends on specific taxa. Our results did not show that the allopolyploid *C. flexuosa* and *C. scutata* were more drought-tolerant than their diploid parents, *C. hirsuta* and *C. parviflora*, respectively. Although our results do not exclude the possibility that these allotetraploids might be more drought-tolerant than diploid F1 hybrids, our data suggest that the differences in ecological preferences of the parental species are likely to be far more important than the effect of polyploidization itself. Therefore, we suggest that the nature of the parental species, rather than genome duplication itself, matters more for ecological speciation by allopolyploid species.

Physiological responses analyzed by gene enrichment analysis

Quantification of gene expression levels using an *Arabidopsis* gene microarray corresponded very well to the qPCR results (Supporting Information Fig. S8 and S9), indicating that the cross-species microarray experiments showed highly reliable results, except for those genes with very low or extremely high induction levels.

Among the GO categories enriched by dry treatment, GO:0009269 (response to desiccation) was shared by *C. hirsuta* and *C. flexuosa*, but not by *C. amara*. On the other hand, other related categories such as GO:0009737 (response to abscisic acid stress), GO:0009738 (abscisic acid-mediated signaling pathway), GO:0009651 (response to salt stress) and GO:0042538 (hyperosmotic salinity response) were shared by all three species. This implies that *C. amara* retains the ability to activate these signaling pathways in response to drought, but cannot confer sufficient stress tolerance. Most probably, the long absence of selective pressure produced by drought stress because of its wet habitat may have impaired the drought response ability, even though *C. amara* can still induce the expression of some drought tolerance-related genes such as the *LEA* family.

On the other hand, only a few GO categories were induced by wet treatment in *C. amara*, consistent with the low number of upregulated genes. This might have been influenced by the bias of the registered GO category, because submergence tolerance is obviously less studied than drought tolerance, hence significantly fewer genes are registered for this phenotype. Thus, only a few GO categories directly related to submergence tolerance can be found, such as GO:0001666 (response to hypoxia, 70 *A. thaliana* genes) and GO:0009413 (response to flooding, only one *A. thaliana* gene). These categories were not listed in all of the three species we studied. Instead of an accumulation of GO categories related to an active response to submergence, many categories related to stress response were found in *C. hirsuta*, such as GO:0000302 (response to reactive oxygen species), GO:0042542 (response to hydrogen peroxide) and GO:0009644 (response to high light intensity). This suggests that the wet treatment increased the stress status of *C. hirsuta*, most probably through reactive oxygen species generated by relatively high amounts of light under limited gas exchange

conditions. Several more categories related to stress were shared by *C. hirsuta* and *C. flexuosa* but not by *C. amara*, such as GO:0009642 (response to light intensity) and GO:0010150 (leaf senescence). All GO categories described above have nominal p-value of zero, expressing an actual p-value of less than 1/1000 by GSEA definition, except for GO:0009644 (response to high light intensity, *C. hirsuta*, p=0.09). In summary, these results suggest that *C. amara* has developed a new mechanism to avoid or reduce oxidative stress caused by submergence, and the same mechanism is working in *C. flexuosa*: not perfectly, but to some extent.

Advantages and disadvantages of allopolyploid species as generalists suggested by the transcriptomic responses to stresses

The results of transcriptome analyses supported the polyploid plasticity hypothesis. Clustering analysis of the transcriptomes showed that the expression pattern of the allotetraploid *C. flexuosa* was similar to that of *C. hirsuta* after the dry treatment and similar to that of *C. amara* after the wet treatment (Fig. 4). This indicates that allopolyploids can take the advantage of utilizing two expression patterns according to environmental changes and have the ability to be generalists in response to hydrological changes. We strongly suggest that this is the major molecular basis of its equivalent growth pattern in fluctuating, submerged and unsubmerged conditions (Fig. 2) as well as in its naturally fluctuating habitats.

Although *C. flexuosa* might appear to be superior to its parents by combining two expression patterns, this species grows in close proximity with *C. amara* or *C. hirsuta* along soil-moisture and waterlogging gradients near streams in Europe (Landolt 2001; Grime *et al.* 2007). This indicates that *C. flexuosa* cannot outcompete *C. amara* or *C. hirsuta*, specialists in submerged and unsubmerged natural habitats, respectively. Thus, the advantage of adapting to fluctuating environments using two transcriptional patterns might have disadvantageous aspects. Allopolyploids represents the status of fixed heterozygosity, thus the expression level of the allopolyploids would be the average of the two parents when *cis*-regulatory differences are dominant. Indeed, we observed the intermediate gene

upregulation level of *C. flexuosa*, between those of its two parents (Table 1). This means that the induction of a large number of stress response genes is lower in the allopolyploid generalist than in diploid specialists, which would attenuate the stress responses.

Many factors have been discussed in the literature as being advantageous or disadvantageous in polyploidy: heterosis, redundancy, change in mating systems are considered advantages in most cases, and changes in cellular architecture, problems in meiosis and mitosis, gene regulatory changes and epistatic instability are considered disadvantages in most cases (Comai 2005; Levin 2002; Stebbins 1971). Our results for *Cardamine* suggest that combining the expression patterns of two parental species confers both advantages and disadvantages on allopolyploid species as discussed above. We suggest that the fixed heterozygosity of allopolyploid species enables the exploitation of two parental expression patterns depending on particular environments and confers the advantage of becoming a generalist and provides the molecular basis of "general purpose genotypes" proposed by Stebbins (1971). However, it also imposes the disadvantage of reduced induction of stress response genes as a trade-off. We suggest that these apply not only to soil moisture and waterlogging responses at a fine scale but also to other stress responses. The allopolyploid *Arabidopsis kamchatica* has a broad distribution in terms of both latitude and altitude (Kenta *et al.* 2011; Shimizu-Inatsugi *et al.* 2009) and inherited its gene expression patterns of cold responses from one of the parents, *A. lyrata*. The expression levels of many genes were intermediate between the two parents, *A. lyrata and A. halleri* (Akama et al. 2014, Paape et al. unpublished).

Recurrent allopolyploid speciation along soil-moisture and waterlogging gradients in Cardamine

The genus *Cardamine* provides a unique opportunity to study recurrent allopolyploid speciation into diverse hydrological niches along soil-moisture and waterlogging gradients. We suggest that the critical innovation in this genus was the exploitation of a submerged habitat with spring flowering by the *C. amara* group. This niche is rarely occupied by other plants because of disadvantages such as submergence, low temperature or difficulties in sexual propagation. The molecular phylogeny of both

nuclear and plastid sequences encompassing diploid species of *Cardamine* supported by high bootstrap values suggests that diploid lineages in a range of unsubmerged environments including *C. parviflora* and C. *hirsuta* split earlier, and then *C. amara* emerged later in the genus (Lihova *et al.* 2006b). Then, *C. amara* hybridized with a number of different *Cardamine* species that grew in a range of unsubmerged conditions. Its hybridization with *C. rivularis* and *C. pratensis* in the past 150 years yielded the polyploids *C. insueta* and *C. schulzii*, which coexist in the small Swiss village of Urnerboden, with documented hydrological differences (Mandakova *et al.* 2013; Urbanska *et al.* 1997; Urbanska-Worytkiewicz & Landolt 1978). *Cardamine amara* was also a parental species of the hexaploid *C. asarifolia*, pentaploid *C. ferrari*, octaploid *C. occulta* (formerly called Asian *C. flexuosa*), (Lihova *et al.* 2006a; Lihova *et al.* 2006b; Marhold *et al.* 2016). Here, we studied two cases: hybridization with a diploid *C. hirsuta* resulting in the allotetraploid *C. flexuosa*, and that with a diploid *C. parviflora* resulting in the allotetraploid *C. scutata*. In both cases, the resultant allopolyploids performed well in fluctuating environments. Interestingly, the two diploid species *C. hirsuta* and *C. parviflora* have clearly different niches among unsubmerged environments in Europe, the former being distributed in drier habitats than the latter (Grime *et al.* 2007; Lihova *et al.* 2006a). Consistent with the parental differences, *C. flexuosa* tends to grow in drier habitats than *C. scutata*, which is found in fairly wet habitats such as paddy fields, where fluctuations in water levels can be extreme (Kimata 1993). This implies that the allopolyploidization of *C. flexuosa* and *C. scutata* resulted in the exploitation of distinct niches thanks to the difference in one parent, although the distribution ranges of *C. flexuosa* and *C. scutata* do not overlap and their ecological niches cannot be compared directly in natural habitats.

In this study, we showed species differences in acute transcriptomic changes following dry and wet treatments. We suggest that this played a major role in niche differentiation, but other traits also undoubtedly contributed. Genes induced by progressive droughts over the course of several weeks are different from those induced by acute droughts, and may be important for long-term survival (Harb *et al.* 2010). The seeds of *C. hirsuta* die when submerged, while the octaploid *C. occulta* (formerly called Asian *C. flexuosa*) requires submergence to break seed dormancy (Yatsu *et al.* 2003). The latter

species showed phenotypic plasticity in diverse traits (Kudoh *et al.* 1995; Kudoh *et al.* 1996). The establishment of polyploids might be enhanced by the evolution of self-compatibility or changes in pathogen resistance (Levin 2002; Menardo *et al.* 2016; Shimizu & Tsuchimatsu 2015). Allopolyploid speciation into a new hydrological niche is probably not unique to *Cardamine*. Contemporary polyploid speciation of *Tragopogon micelles* and *T. mirus* might also represent niche differentiation in water usages (Levin 2002; Novak *et al.* 1991).

Recurrent allopolyploid speciation of genus Cardamine *as a model to study ecological speciation and adaptive radiation into hydrological niches*

Ecological speciation needs the concomitant establishment of both ecological niche divergence and reproductive isolation without geographic isolation (Nosil 2012). The study of ecological speciation in plants has focused on the evolution of flower color as a "magic trait," which can be associated with changes in the interaction with pollinators and thus allows niche separation and reproductive isolation simultaneously (Nosil 2012; Waser & Campbell 2004). Here, we propose that allopolyploid speciation into hydrological niches provides an attractive model to study ecological speciation in plants. First, allopolyploidization confers a major effect on soil-moisture and waterlogging responses instantaneously, and enables the new polyploid species to live in a new ecological niche among a large number of different niches in steep gradients over a short distance (Silvertown *et al.* 1999). Second, the difference in ploidy confers strong reproductive isolation instantaneously. This would be essential for a new species to persist against its disadvantage of having a minority cytotype, because allopolyploidization cannot occur allopatrically, so pollination among plants in different niches along the fine-scale gradients would occur easily.

In contrast to speciation by lineage splitting, which typically occurs gradually, hybridization events can confer an instant major effect on the ecological niche and reproductive isolation. More importantly, parental species or close relatives are often known in the case of allopolyploid speciation, and thus their ecology can be studied experimentally, while it is not trivial to infer the ecology of

common ancestor of sibling species (for example, the common ancestors of human beings and chimpanzees were not chimpanzees). Here, we took advantage of the availability of parental species for growth experiments and transcriptomic studies. Although evolutionary changes in polyploid as well as diploids after the allopolyploidization events can play a significant role, several allopolyploid speciation events occurred during the past 150 years (Urbanska *et al.* 1997), indicating that ecological differentiation can occur immediately.

A general definition of adaptive radiation is "the rise of a diversity of ecological roles and attendant adaptations in different species," i.e., recurrent ecological speciation, within a lineage (Givnish 1997; Rundell & Price 2009). Being one of the largest genera in the Brassicaceae and with previous reports on hydrological niche segregation patterns (Urbanska-Worytkiewicz & Landolt 1978), we suggest that recurrent allopolyploidization in *Cardamine* represents an adaptive radiation to diverse hydrological niches. Soil-moisture and waterlogging gradients provide a large number of different niches (Silvertown *et al.* 1999), and we emphasize that the niches should not be considered static, but that fluctuations in water levels will amplify the diversity of hydrological niches. Thus, allopolyploids did not just occupy intermediate niches, but were also able to exploit new fluctuating environments. The importance of hybridization in adaptive radiation or ecological speciation was suggested for example in cichlids and Hawaiian Silverswords, in that initial hybridization (or allopolyploidization) could have stimulated subsequent rapid speciation by producing novel hybrid genomes (Lawton-Rauh *et al.* 2003; Seehausen 2004). In contrast, a recent meta-analysis of diverse lineages revealed that polyploidization events themselves did not enhance the subsequent speciation rate on average, but rather lowered it (Mayrose *et al.* 2011). We propose that *Cardamine* represents another novel mechanism behind adaptive radiation, i.e. recurrent allopolyploidization with various combinations of parents, and thus reconciles the controversy regarding the role of polyploidization in species diversification. That is, recurrent polyploidization events, rather than diversification after polyploidization, have played a major role in ecological speciation. This does not exclude further diversification of polyploid lineages (potentially the case of *C. scutata* and *C. niigatensis*) (Lihova et al. 2006a). A more specific definition of adaptive radiation entails rapidly multiplying lineages

(Rundell & Price 2009). Allopolyploid speciation events occur quickly in a generation. Indeed, *C. insueta* and *C. schulzii* have speciated in the past 150 years (Urbanska *et al.* 1997), and *C. flexuosa* is also regarded as a relatively recent allopolyploid (10^4 to 10^5 years old) (Mandakova *et al.* 2014). The upper time boundary of the recurrent allopolyploidization in *Cardamine* is likely to be the recent split of *C. amara*, which emerged after the split of other diploid *Cardamine* species (see above). There is ongoing controversy regarding evolutionary rates and lineage splitting times in the Brassicaceae (Beilstein *et al.* 2010; Hohmann *et al.* 2015; Koch *et al.* 2000; Mandakova *et al.* 2014; Ossowski *et al.* 2010; Shimizu & Tsuchimatsu 2015). Thus, quantitative studies are essential to obtain more rigorous estimates of the speed of diversification in *Cardamine*. Current data suggest that after a relatively slow diversification of unsubmerged diploid species, the adaptation of *C. amara* into submerged environments would have triggered a burst of allopolyploid speciation into diverse hydrological niches. Geographically, there are multiple centers of high species richeness in *Cardamine* (e.g., about 70 species in Far East and Himalayas, about 49 species in European Mediterranean and Caucasus, about 50 species in North and Central America, 20-25 species in New Zealand) (Lihova & Marhold 2006). So it is possible that adaptive radiation occurred independently in each region, although detailed analysis would be necessary to test it because the current distributions of polyploids and diploids may be poor indicators of the geographic origin of polyploidy (Levin 2002).

Implications of the transcriptomic studies and future possibilities

Here, we studied the sum expression rates of two homeologs because this matters functionally in the vast majority of homeologous pairs except for the cases in which the proteins coded by the homeologs gained different functions. Clustering analysis of genes with significant changes in levels of expression in response to the wet or dry treatments showed that *C. flexuosa* mimics either of its parents, depending on the condition (Fig. 4). However, there are many genes that are distinct from the parental pattern, which could be termed novel expression patterns. In the Venn diagrams shown in Fig. 3, more than two-thirds of the genes that were induced in *C. flexuosa* were shared with parents in

both dry and wet treatments; however, many genes were upregulated only in *C. flexuosa*. In addition, by rank order analysis, the expression levels of approximately half of the top 500 genes were not intermediate in *C. flexuosa* (Table 1). These findings suggest that the expression levels of many *C. flexuosa* genes are not merely the average of two parents, as would be expected by cis-regulation alone. They could have arisen from epistatic interactions (or trans-regulations) or evolutionary changes in allopolyploids after gene networks had merged through polyploidization events, as well as in diploids (Soltis *et al.* 2014; Song & Chen 2015; Yoo *et al.* 2014). The present study focused on the hierarchical clustering of gene expression levels with conserved probe sequences for microarray analysis, thus those homeologous pairs with high sequence divergence affecting hybridization to the arrays were not analyzed. To assess the transcriptional regulations including diverged homeologous pairs, it will be important to obtain the expression levels of each homeolog separately using RNA sequencing approaches. A number of bioinformatic workflows (Akama *et al.* 2014; Page *et al.* 2013) are being developed for polyploid transcriptomes.

Phenotypic plasticity is defined as the ability of a single genotype to produce a range of phenotypes in different environments, and thus the framework of phenotypic plasticity has been recently applied to transcriptomics (Del Santo *et al.* 2013; Zhou *et al.* 2012). The clustering suggested that the allopolyploid *C. flexuosa* has a transcriptomic plasticity depending on the wet and dry conditions. Transcriptomic plasticity of polyploids may facilitate the phenotypic plasticity in morphological and life history traits (in the octaploid *C. occulta* described as Asian *C. flexuosa*) (Kudoh *et al.* 1995; Kudoh *et al.* 1996).

More importantly, transcriptomic studies in naturally fluctuating environments (termed *in natura*) are starting to show that the transcriptomic patterns might be highly different from those seen in laboratory studies, although laboratory experiments can be useful to control each environmental parameter separately (Alvarez *et al.* 2015; Kobayashi *et al.* 2013; Kudoh 2015; Shimizu *et al.* 2011). Our growth experiments supported the importance of environmental fluctuations in *Cardamine* allopolyploids, and the availability of parental species in polyploid speciation (discussed above) would facilitate such transcriptomic studies.

Acknowledgements

The authors thank Reiko Akiyama for valuable discussions, Karol Marhold and Judita Lihova for sample collection, and Andrea Patrignani at the Functional Genomics Center of Zurich for his support of microarray experiments. The study was supported by Swiss National Science Foundation, Grant-in-Aid for Scientific Research on Innovative Areas 'Correlative Gene System' of MEXT, Japan, to KKS; by the University Research Priority Program of Evolution in Action and Systems Biology/Functional Genomics of the University of Zurich to KKS and RSI; by a Young Investigator Award of Human Frontier Science Program to KKS and JS; by Grants-in-Aid for Special Research on Priority Areas on Genome Barriers in Plant Reproduction from MEXT, Japan, to HK and KKS; by a Marie-Heim Hoegtlin grant from the Swiss National Science Foundation and Forschungskredit of the University of Zurich to RSI; by SPIRITS of Kyoto University to HK, KKS and RSI; by the Japan Society for the Promotion of Science (JSPS) KAKENHI Grants 25128704 and 15H01717 to JS; and by a grant from the Japan Science and Technology Agency (JST) PRESTO to AT. A supercomputing resource was provided by the National Institute of Genetics, Research Organization of Information and Systems, Shizuoka, Japan.

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Data Accessibility

Gene expression data used in this paper is accessible in the gene expression omnibus (GSE77673).

Author Contributions

RSI, HK, JS and KKS designed research, RSI and KH performed experiments, AT and JS analyzed transcriptome data with inputs from RSI and KKS, RSI and KKS wrote the paper with inputs from all others.

Figure captions

Fig. 1 Images of the plants in their typical habitats and their relationships. Two trios of parental diploid and allotetraploid species are shown in their representative habitats. Gray bars indicate the parental relationships among species.

Fig. 2 Leaf numbers of the diploid and allotetraploid species in growth experiments. Two trios of parental diploid and allotetraploid species were grown in three different hydration conditions: unsubmerged (Un-subm.), fluctuating (Fluct.) and submerged (Subm.) for 10 weeks. The first trio is **A.** *C. amara* **B.** *C. hirsuta* and **C.** *C. flexuosa*, and the second trio is **D.** *C. amara*, **E.** *C. parviflora* and **F.** *C. scutata*. The data shown in box plots were analyzed using Tukey's HSD test. Combinations with significant difference between conditions are indicated by: $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Fig. 3 Venn diagrams of the genes differentially expressed after dry or wet treatments using microarray. The numbers of genes upregulated (Up) or downregulated (Down) 4-fold by the dry treatment are shown on the left, and the numbers of genes upregulated or downregulated 3-fold by the wet treatment are shown on the right for each species. Percentage in brackets indicates the proportion in the total upregulated or downregulated genes in three species.

Fig. 4 Hierarchical clustering of genes with statistically significant changes in expression levels produced by dry or wet treatments. Genes with significant expression level change were extracted as described in Methods and the resulting genes were analyzed using Ward's hierarchical clustering method with the Euclid distance. The percentages show the results of bootstrap test.

Supporting Information List

Table S1 Origins of plants used in this paper.

Table S2 The expression level change of all analyzed genes in $\log_2$-fold.

Table S3-A The genes used for clustering with significant change by dry treatment.

Table S3-B The genes used for clustering with significant change by wet treatment.

Table S4 The primers used for qPCR.

Table S5 GO categories of genes upregulated after dry treatment by GSEA analysis.

Table S6 GO categories of genes upregulated after wet treatment by GSEA analysis.

Fig. S1 Pairwise plots of the microarray expression data between biological replicates in each species and treatment condition.

Fig. S2 Longest leaf length of the diploids (*C. amara* and *C. hirsuta*) and allotetraploid (*C. flexuosa*) in growth experiments.

Fig. S3 Rosette width of the diploids (*C. amara* and *C. hirsuta*) and allotetraploid (*C. flexuosa*) in growth experiments.

Fig. S4 Total biomass of the diploids (*C. amara* and *C. hirsuta*) and allotetraploid (*C. flexuosa*) in growth experiments.

Fig. S5 Longest leaf length of the diploids (*C. amara* and *C. parviflora*) and allotetraploid (*C. scutata*) in growth experiments.

Fig. S6 Rosette width of the diploids (*C. amara* and *C. parviflora*) and allotetraploid (*C. scutata*) in growth experiments.

Fig. S7 Total biomass of the diploids (*C. amara* and *C. parviflora*) and allotetraploid (*C. scutata*) in growth experiments.

Fig. S8 The validation of microarray data using qPCR.

Fig. S9 The comparison of gene induction levels by microarray and qPCR.

Fig. S10 Venn diagrams of the enriched GO categories by dry and wet treatment.

Table 1 The categorization of the top 500 up-regulated genes by dry (A) or wet (B) treatment.

A. Top 500 genes by dry treatment						
C. amara > *C. flexuosa* > *C. hirsuta*	88		*C. flexuosa* > *C. amara* > *C. hirsuta*	34	*C. amara* > *C. hirsuta* > *C. flexuosa*	76
C. hirsuta > *C. flexuosa* > *C. amara*	178		*C. flexuosa* > *C. hirsuta* > *C. amara*	44	*C. hirsuta* > *C. amara* > *C. flexuosa*	80
sum	266		sum	78	sum	156
B. Top 500 genes by wet treatment						
C. amara > *C. flexuosa* > *C. hirsuta*	94		*C. flexuosa* > *C. amara* > *C. hirsuta*	37	*C. amara* > *C. hirsuta* > *C. flexuosa*	46
C. hirsuta > *C. flexuosa* > *C. amara*	208		*C. flexuosa* > *C. hirsuta* > *C. amara*	59	*C. hirsuta* > *C. amara* > *C. flexuosa*	56
sum	302		sum	96	sum	102